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(21) International Application Number: PCT/CA96/00398 (22) International Filing Date: 7 June 1996 (07.06.96) (30) Priority Data: 08/476,397 7 June 1995 (07.06.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/476,397 (CIP) Filed on 7 June 1995 (07.06.95) (71) Applicant (for all designated States except US): CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Av- enue West, North York, Ontario M2R 3T4 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): LI, Xiaomao [CA/CA]; 106 Glenmanor Way, Thornhill, Ontario L4J 3E5 (CA). EWASYSHYN, Mary, E. [CA/CA]; Apartment 1506, 120 Torresdale, Willowdale, Ontario M2R 3N7 (CA). SAMBHARA, Suryaprakash [CA/CA]; 50 Harness Circle, Markham, Ontario L3S 1Y1 (CA). KLEIN, Michel, H. [CA/CA]; 16 Munro Boulevard, Willowdale, Ontario M2P 1B9 (CA).	(74) Agent: STEWART, Michael, I; Sim & McBurney, 6th floor, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published Without international search report and to be republished upon receipt of that report.	
(54) Title: NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES (57) Abstract <p>Vectors containing a nucleotide sequence coding for an F protein of respiratory syncytial virus (RSV) and a promoter for such sequence, preferably a cytomegalovirus promoter, are described. Such vectors also may contain a further nucleotide sequence located adjacent to the RSV F protein encoding sequence to enhance the immunoprotective ability of the RSV F protein when expressed <i>in vivo</i>. Such vectors may be used to immunize a host, including a human host, by administration thereto. Such vectors also may be used to produce antibodies for detection of RSV infection in a sample.</p>		

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NUCLEIC ACID RESPIRATORY SYNCYTIAL VIRUS VACCINES

FIELD OF INVENTION

The present invention is related to the field of Respiratory Syncytial Virus (RSV) vaccines and is particularly concerned with vaccines comprising nucleic acid sequences encoding the fusion (F) protein of RSV.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States Patent Application No. 08/476,397, filed June 7, 1995.

BACKGROUND OF INVENTION

Respiratory syncytial virus (RSV), a negative-strand RNA virus belonging to the Paramyxoviridae family of viruses, is the major viral pathogen responsible for bronchiolitis and pneumonia in infants and young children (ref. 1 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Acute respiratory tract infections caused by RSV result in approximately 90,000 hospitalizations and 4,500 deaths per year in the United States (ref. 2). Medical care costs due to RSV infection are greater than \$340 M annually in the United States alone (ref. 3). There is currently no licensed vaccine against RSV. The main approaches for developing an RSV vaccine have included inactivated virus, live-attenuated viruses and subunit vaccines.

The F protein of RSV is considered to be one of the most important protective antigens of the virus. There is a significant similarity (89% identity) in the amino acid sequences of the F proteins from RSV subgroups A and

B (ref. 3) and anti-F antibodies can cross-neutralize viruses of both subgroups as well as protect immunized animals against infection with viruses from both subgroups (ref. 4). Furthermore, the F protein has been identified as a major target for RSV-specific cytotoxic T-lymphocytes in mice and humans (ref. 3 and ref. 5).

The use of RSV proteins as vaccines may have obstacles. Parenterally administered vaccine candidates have so far proven to be poorly immunogenic with regard to the induction of neutralizing antibodies in seronegative humans or chimpanzees. The serum antibody response induced by these antigens may be further diminished in the presence of passively acquired antibodies, such as the transplacentally acquired maternal antibodies which most young infants possess. A subunit vaccine candidate for RSV consisting of purified fusion glycoprotein from RSV infected cell cultures and purified by immunoaffinity or ion-exchange chromatography has been described (ref. 6). Parenteral immunization of seronegative or seropositive chimpanzees with this preparation was performed and three doses of 50 μ g were required in seronegative animals to induce an RSV serum neutralizing titre of approximately 1:50. Upon subsequent challenge of these animals with wild-type RSV, no effect of immunization on virus shedding or clinical disease could be detected in the upper respiratory tract. The effect of immunization with this vaccine on virus shedding in the lower respiratory tract was not investigated, although this is the site where the serum antibody induced by parenteral immunization may be expected to have its greatest effect. Safety and immunogenicity studies have been performed in a small number of seropositive individuals. The vaccine was found to be safe in seropositive children and in three seronegative children (all > 2.4 years of age). The effects of immunization on lower respiratory tract

disease could not be determined because of the small number of children immunized. One immunizing dose in seropositive children induced a 4-fold increase in virus neutralizing antibody titres in 40 to 60% of the vaccinees. Thus, insufficient information is available from these small studies to evaluate the efficacy of this vaccine against RSV-induced disease. A further problem facing subunit RSV vaccines is the possibility that inoculation of seronegative subjects with immunogenic preparations might result in disease enhancement (sometimes referred to as immunopotential), similar to that seen in formalin inactivated RSV vaccines. In some studies, the immune response to immunization with RSV F protein or a synthetic RSV FG fusion protein resulted in a disease enhancement in rodents resembling that induced by a formalin-inactivated RSV vaccine. The association of immunization with disease enhancement using non-replicating antigens suggests caution in their use as vaccines in seronegative humans.

Live attenuated vaccines against disease caused by RSV may be promising for two main reasons. Firstly, infection by a live vaccine virus induces a balanced immune response comprising mucosal and serum antibodies and cytotoxic T-lymphocytes. Secondly, infection of infants with live attenuated vaccine candidates or naturally acquired wild-type virus is not associated with enhanced disease upon subsequent natural reinfection. It will be challenging to produce live attenuated vaccines that are immunogenic for younger infants who possess maternal virus-neutralizing antibodies and yet are attenuated for seronegative infants greater than or equal to 6 months of age. Attenuated live virus vaccines also have the risks of residual virulence and genetic instability.

Injection of plasmid DNA containing sequences encoding a foreign protein has been shown to result in

expression of the foreign protein and the induction of antibody and cytotoxic T-lymphocyte responses to the antigen in a number of studies (see, for example, refs. 7, 8, 9). The use of plasmid DNA inoculation to express viral proteins for the purpose of immunization may offer several advantages over the strategies summarized above. Firstly, DNA encoding a viral antigen can be introduced in the presence of antibody to the virus itself, without loss of potency due to neutralization of virus by the antibodies. Secondly, the antigen expressed *in vivo* should exhibit a native conformation and, therefore, should induce an antibody response similar to that induced by the antigen present in the wild-type virus infection. In contrast, some processes used in purification of proteins can induce conformational changes which may result in the loss of immunogenicity of protective epitopes and possibly immunopotentiality. Thirdly, the expression of proteins from injected plasmid DNAs can be detected *in vivo* for a considerably longer period of time than that in virus-infected cells, and this has the theoretical advantage of prolonged cytotoxic T-cell induction and enhanced antibody responses. Fourthly, *in vivo* expression of antigen may provide protection without the need for an extrinsic adjuvant.

The ability to immunize against disease caused by RSV by administration of a DNA molecule encoding an RSV F protein was unknown before the present invention. In particular, the efficacy of immunization against RSV induced disease using a gene encoding a secreted form of the RSV F protein was unknown. Infection with RSV leads to serious disease. It would be useful and desirable to provide isolated genes encoding RSV F protein and vectors for *in vivo* administration for use in immunogenic preparations, including vaccines, for protection against disease caused by RSV and for the generation of diagnostic reagents and kits. In particular, it would be

desirable to provide vaccines that are immunogenic and protective in humans, including seronegative infants, that do not cause disease enhancement (immunopotentialiation).

5

SUMMARY OF INVENTION

The present invention relates to a method of immunizing a host against disease caused by respiratory syncytial virus, to nucleic acid molecules used therein, and to diagnostic procedures utilizing the nucleic acid molecules. In particular, the present invention is directed towards the provision of nucleic acid respiratory syncytial virus vaccines.

In accordance with one aspect of the invention, there is provided a vector, comprising:

15

a first nucleotide sequence encoding an RSV F protein or a protein capable of inducing antibodies that specifically react with RSV F protein;

a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein, and

20

a second nucleotide sequence located adjacent the first nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed in vivo from the vector in a host.

25

The first nucleotide sequence may be that which encodes a full-length RSV F protein, as seen in Figure 2 (SEQ ID No: 2). Alternatively, the first nucleotide sequence may be that which encodes an RSV F protein from which the transmembrane region is absent. The latter embodiment may be provided by a nucleotide sequence which encodes a full-length RSV F protein but contains a translational stop codon immediately upstream of the start of the transmembrane coding region, thereby preventing expression of a transmembrane region of the RSV F protein, as seen in Figure 3 (SEQ. ID No. 4). The lack of expression of the transmembrane region results in

30

35

a secreted form of the RSV F protein.

The second nucleotide sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes the RSV protein. Such second nucleotide sequence may be located between the first nucleotide sequence and the promoter sequence. Such second nucleotide sequence may be that of rabbit β -globin intron II, as shown in Figure 8 (SEQ ID No: 5).

A vector encoding the F protein and provided by this aspect of the invention may specifically be pXL2 or pXL4, as seen in Figures 5 or 7.

The promoter sequence may be an immediate early cytomegalovirus (CMV) promoter. Such cytomegalovirus promoter has not previously been employed in vectors containing nucleotide sequences encoding an RSV F protein.

Accordingly, in another aspect of the invention, there is provided a vector, comprising:

- a first nucleotide sequence encoding an RSV F protein or a protein capable of generating antibodies that specifically react with RSV F protein, and
- a cytomegalovirus promoter operatively coupled to the first nucleotide sequence for expression of the RSV F protein.

The first nucleotide sequence may be any of the alternatives described above. The second nucleotide sequence, included to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* from the vector in a host, described above also may be present located adjacent a first nucleotide sequence in a vector provided in accordance with this second aspect of the invention.

Certain of the vectors provided herein may be used to immunize a host against RSV infection or disease by *in vivo* expression of RSV F protein lacking a transmembrane

region following administration of the vectors. In accordance with a further aspect of the present invention, therefore, there is provided a method of immunizing a host against disease caused by infection with respiratory syncytial virus, which comprises administering to the host an effective amount of a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host, which may be a human. The promoter may be an immediate early cytomegalovirus promoter.

15 The nucleotide sequence encoding the truncated RSV F protein lacking the transmembrane region may be that as described above.

A vector containing a second nucleotide sequence located adjacent a first nucleotide sequence encoding an RSV F protein, a protein capable of inducing antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region and effective to enhance the immunoprotective ability of the RSV F protein expressed by the first nucleotide sequence may be used to immunize a host. Accordingly, in an additional aspect of the present invention, there is provided a method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to the host an effective amount of a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region, a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein, and a second nucleotide sequence located adjacent the first sequence to enhance

the immunoprotective ability of the RSV-F protein when expressed in vivo from said vector in said host. Specific vectors which may be used in this aspect of the invention are those identified as pXL2 and pXL4 in
5 Figures 5 and 7.

The present invention also includes a novel method of using a gene encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a
10 transmembrane region to protect a host against disease caused by infection with respiratory syncytial virus, which comprises:

isolating the gene;
operatively linking the gene to at least one control
15 sequence to produce a vector, said control sequence directing expression of the RSV F protein when said vector is introduced into a host to produce an immune response to the RSV F protein, and
introducing the vector into the host.

20 The procedure provided in accordance with this aspect of the invention may further include the step of:

operatively linking the gene to an immunoprotection
enhancing sequence to produce an enhanced
immunoprotection by the RSV F protein in the host,
25 preferably by introducing the immunoprotection enhancing sequence between the control sequence and the gene.

In addition, the present invention includes a method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial
30 virus, which comprises:

isolating a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region;

35 operatively linking the first nucleotide sequence to at least one control sequence to produce a vector, the

control sequence directing expression of the RSV F protein when introduced into a host to produce an immune response to the RSV F protein when expressed in vivo from the vector in a host, and

- 5 formulating the vector as a vaccine for in vivo administration.

The first nucleotide sequence further may be operatively linked to a second nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed in vivo from the vector in a host. The vector may be selected from pXL1, pXL2 and pXL4. The invention further includes a vaccine for administration to a host, including a human host, produced by this method as well as immunogenic compositions comprising an immunoeffective amount of the vectors described herein.

As noted previously, the vectors provided herein are useful in diagnostic applications. In a further aspect of the invention, therefore, there is provided a method of determining the presence of an RSV F protein in a sample, comprising the steps of:

- (a) immunizing a host with a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to the first nucleotide sequence for expression of the RSV F protein in the host to produce antibodies specific for the RSV F protein;
- (b) isolating the RSV F protein specific antibodies;
- (c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and the RSV F protein-specific antibodies; and
- (d) determining production of the complexes.

The vector employed to elicit the antibodies may be pXL1, pXL2, pXL3 or pXL4.

The invention also includes a diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

- (a) a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein, or a RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in a host immunized therewith to produce antibodies specific for the RSV F protein;
- (b) isolation means to isolate said RSV F protein specific antibodies;
- (c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies; and
- (d) identifying means to determine production of the complex.

The present invention is further directed to immunization wherein the polynucleotide is an RNA molecule which codes for an RSV F protein, a protein capable of inducing antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region.

The present invention is further directed to a method for producing RSV F protein specific polyclonal antibodies comprising the use of the immunization method described herein, and further comprising the step of isolating the RSV F protein specific polyclonal antibodies from the immunized animal.

The present invention is also directed to a method for producing monoclonal antibodies specific for an F

protein of RSV, comprising the steps of:

- 5 (a) constructing a vector comprising a first nucleotide sequence encoding a RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,
 - 10 a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from said vector in a host.
- (b) administering the vector to at least one mouse to produce at least one immunized mouse;
- (c) removing B-lymphocytes from the at least one immunized mouse;
- 15 (d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;
- (e) cloning the hybridomas;
- (f) selecting clones which produce anti-F protein antibody;
- 20 (g) culturing the anti-F protein antibody-producing clones; and
- (h) isolating anti-F protein monoclonal antibodies.

25 In this application, the term "RSV F protein" is used to define a full-length RSV F protein, such proteins having variations in their amino acid sequences including those naturally occurring in various strains of RSV, a secreted form of RSV F protein lacking a transmembrane region, as well as functional analogs of the RSV F protein. In this application, a first protein is a
30 "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a
35 substitution, addition or deletion mutant thereof.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following General Description and Examples with reference to the Figures in which:

5 Figure 1 illustrates a restriction map of the gene encoding the F protein of Respiratory Syncytial Virus;

 Figure 2 illustrates the nucleotide sequence of the gene encoding the membrane attached form of the F protein of Respiratory Syncytial Virus (SEQ ID No: 1) as well as
10 the amino acid sequence of the RSV F protein encoded thereby (SEQ ID No: 2);

 Figure 3 illustrates the nucleotide sequence of the gene encoding the secreted form of the RSV F protein lacking the transmembrane region (SEQ ID No: 3) as well
15 as the amino acid sequence of the truncated RSV F protein lacking the transmembrane region encoded thereby (SEQ ID No: 4);

 Figure 4 shows the construction of plasmid pXL1 containing the gene encoding a secreted form of the RSV
20 F protein lacking the transmembrane region

 Figure 5 shows the construction of plasmid pXL2 containing a gene encoding a secreted form of the RSV F protein lacking the transmembrane region and containing the rabbit β -globin Intron II sequence;

25 Figure 6 shows the construction of plasmid pXL3 containing the gene encoding a full length membrane attached form of the RSV F protein;

 Figure 7 shows the construction of plasmid pXL4 containing a gene encoding a membrane attached form of the RSV F protein and containing the rabbit β -globin
30 Intron II sequence; and

 Figure 8 shows the nucleotide sequence for the rabbit β -globin Intron II sequence (SEQ ID No. 5).

GENERAL DESCRIPTION OF INVENTION

As described above, the present invention relates generally to polynucleotide, including DNA, immunization to obtain protection against infection by respiratory syncytial virus (RSV) and to diagnostic procedures using particular vectors. In the present invention, several recombinant vectors were constructed to contain a nucleotide sequence encoding an RSV F protein.

The nucleotide sequence of the full length RSV F gene is shown in Figure 2 (SEQ ID No: 1). Certain constructs provided herein include the nucleotide sequence encoding the full-length RSV F (SEQ ID No: 2) protein while others include an RSV F gene modified by insertion of termination codons immediately upstream of the transmembrane coding region (see Figure 3, SEQ ID No: 3), to prevent expression of the transmembrane portion of the protein and to produce a secreted or truncated RSV F protein lacking a transmembrane region (SEQ ID No. 4).

The nucleotide sequence encoding the RSV F protein is operatively coupled to a promoter sequence for expression of the encoded RSV F protein. The promoter sequence may be the immediately early cytomegalovirus (CMV) promoter. This promoter is described in ref. 13. Any other convenient promoter may be used, including constitutive promoters, such as, Rous Sarcoma Virus LTRs, and inducible promoters, such as metallothionine promoter, and tissue specific promoters.

The vectors provided herein, when administered to an animal, effect in vivo RSV F protein expression, as demonstrated by an antibody response in the animal to which it is administered. Such antibodies may be used herein in the detection of RSV protein in a sample, as described in more detail below. When the encoded RSV F protein is in the form of an RSV F protein from which the transmembrane region is absent, such as plasmid pXL1 (Figure 4), the administration of the vector conferred

protection in mice and cotton rats to challenge by live RSV virus neutralizing antibody and cell mediated immune responses and an absence of immunopotential in immunized animals, as seen from the Examples below.

5 The recombinant vector also may include a second nucleotide sequence located adjacent the RSV F protein encoding nucleotide sequence to enhance the immunoprotective ability of the RSV F protein when expressed *in vivo* in a host. Such enhancement may be provided by increased *in vivo* expression, for example, by increased mRNA stability, enhanced transcription and/or translation. This additional sequence preferably is located between the promoter sequence and the RSV F protein-encoding sequence.

10 This enhancement sequence may comprise a pair of splice sites to prevent aberrant mRNA splicing during transcription and translation so that substantially all transcribed mRNA encodes an RSV F protein. Specifically, rabbit β -globin Intron II sequence shown in Figure 7 (SEQ ID No: 5) may provide such splice sites, as also described in ref. 15.

15 The constructs containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the truncated RSV F protein lacking a transmembrane region, i.e. plasmid pXL2 (Figure 5), induced complete protection in mice against challenge with live RSV, as seen in the Examples below. In addition, the constructs containing the Intron II sequence, CMV promoter and nucleotide sequence coding for the full-length RSV F protein, i.e. plasmid pXL4 (Figure 7), also conferred protection in mice to challenge with live RSV, as seen from the Examples below.

20 The vector provided herein may also comprise a third nucleotide sequence encoding a further antigen from RSV, an antigen from at least one other pathogen or at least one immunomodulating agent, such as cytokine. Such

vector may contain said third nucleotide sequence in a chimeric or a bicistronic structure. Alternatively, vectors containing the third nucleotide sequence may be separately constructed and coadministered to a host, with the nucleic acid molecule provided herein.

The vector may further comprise a nucleotide sequence encoding a heterologous signal peptide, such as human tissue plasminogen activator (TPA), in place of the endogenous signal peptide.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis and treatment of RSV infections. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the RSV F genes and vectors as disclosed herein. The vaccine elicits an immune response in a subject which includes the production of anti- γ antibodies. Immunogenic compositions, including vaccines, containing the nucleic acid may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 9324640, ref. 17) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses

the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a phospholipid/glycolipid/polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactide-co-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain an absorption enhancer.

The RSV F genes and vectors may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and

combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof.

5 Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intravenously, intradermally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic. Alternatively, the immunogenic compositions
10 formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes.
15 Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients,
20 such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be
25 therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize the RSV F protein and antibodies thereto, and if needed, to produce
30 a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 μ g to about 1
35 mg of the RSV F genes and vectors. Suitable regimes for initial administration and booster doses are also

variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which
5 protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or
10 from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered
15 saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to
20 cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to,
25 for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum
30 hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune
35 responses to antigens. These include saponins complexed to membrane protein antigens to produce immune

stimulating complexes (ISCOMS), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as monophoryl lipid A, QS 21 and polyphosphazene.

In particular embodiments of the present invention, the vector comprising a first nucleotide sequence encoding an F protein of RSV may be delivered in conjunction with a targeting molecule to target the vector to selected cells including cells of the immune system.

The polynucleotide may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 10) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 11) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

2. Immunoassays

The RSV F genes and vectors of the present invention are useful as immunogens for the generation of anti-F antibodies for use in immunoassays, including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art. In ELISA assays, the vector first is administered to a host to generate antibodies specific to the RSV F protein. These RSV F-specific antibodies are immobilized onto a selected surface, for example, a surface capable of binding the antibodies, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed antibodies, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites

shown schematically in Figures 4 to 7) in which cDNA encoding the RSV-F was subcloned downstream of the immediate-early promoter, enhancer and intron A sequences of human cytomegalovirus (CMV) and upstream of the bovine growth hormone (BGH) poly-A site. The 1.6 Kb Sspl-PstI fragment containing the promoter, enhancer and intron A sequences of CMV Towne strain were initially derived from plasmid pRL43a obtained from Dr. G.S. Hayward of Johns Hopkins University (ref. 20) and subcloned between EcoRV and PstI sites of pBluescript 11 SK +/- (Stratagene). For the construction of plasmids expressing the secretory form of the F protein (pXL1 and pXL2 in Figs. 4 and 5), the 1.6 Kb EcoRI-BamHI fragment containing the truncated form of the F cDNA originally cloned from a clinical isolate belonging to subgroup A was excised from pRSVF (ref. 18 and WO 93/14207) and subcloned between EcoRI and BamHI sites of pSG5 (Stratagene, ref. 14). Either the 1.6 kb EcoRI-BamHI fragment or the 2.2 kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The 0.6 kb ClaI-EcoRI fragment derived from pSG5 contained the intron II sequences from rabbit β -globin. Subsequently, the plasmids were digested with HindIII, filled-in with Klenow, and digested with XbaI to yield either a 3.2 or a 3.8 Kb fragment. These fragments were used to replace the 0.8 kb NruI-XbaI fragment containing the CMV promoter in pRc/CMV (Invitrogen), resulting in the final pXL1 and pXL2 constructs, respectively.

For the construction of plasmids expressing the full-length F protein (pXL3 and pXL4 - Figs. 6 and 7), the full length RSV F cDNA was excised as a 1.9 kb EcoRI fragment from a recombinant pBluescript M13-SK (Stratagene) containing the insert (ref. 18 and WO 93/14207) and subcloned at the EcoRI site of pSG5

(Stratagene). Either the 1.9 Kb EcoRI fragment or the 2.5 Kb ClaI-BamHI fragment was then excised from the pSG5 construct, filled-in with Klenow and subcloned at the SmaI site of the pBluescript II SK +/- construct containing the promoter and intron A sequences. The rest of the construction for pXL3 and pXL4 was identical to that for pXL1 and pXL2, as described above. Therefore, except for the CMV promoter and intron A sequences, the rest of the vector components in pXL1-4 were derived from plasmid pRc/CMV. Plasmids pXL1 and pXL2 were made to express a truncated/secretory form of the F protein which carried stop codons resulting in a C-terminal deletion of 48 amino acids including the transmembrane (TM) and the C-terminal cytosolic tail as compared to the intact molecule. In contrast, pXL3 and pXL4 were made to express the intact membrane-attached form of the RSV F molecule containing the TM and the cytosolic C-terminal tail. The rationale for the presence of the intron II sequences in pXL2 and pXL4 was that this intron was reported to mediate the correct splicing of RNAs. Since mRNA for the RSV-F has been suspected to have a tendency towards aberrant splicing, the presence of the intron II sequences might help to overcome this. All four plasmid constructs were confirmed by DNA sequencing analysis.

Plasmid DNA was purified using plasmid mega kits from Qiagen (Chatsworth, CA, USA) according to the manufacturer's instructions.

Example 2

This Example describes the immunization of mice. Mice are susceptible to infection by RSV as described in ref. 16.

For intramuscular (i.m) immunization, the anterior tibialis anterior muscles of groups of 9 BALB/c mice (male, 6-8 week old) (Jackson Lab., Bar Harbor, ME, USA) were bilaterally injected with 2 x 50 μ g (1 μ g/ μ L in PBS) of pXL1-4, respectively. Five days prior to DNA

responder cells were incubated with 2×10^3 target cells at varying effector to target ratios in 200 μ L in 96-well V-bottomed tissue-culture plates for 4 hr at 37°C. Spontaneous and total chromium releases were determined by incubating target cells with either medium or 2.5% Triton-X 100 in the absence of responder lymphocytes. Percentage specific chromium release was calculated as $(\text{counts-spontaneous counts})/(\text{total counts-spontaneous counts}) \times 100$. Tests were performed in triplicates and data are expressed as the means of three determinations. For antibody blocking studies in CTL assays, the effector cells were incubated for 1 hr with 10 μ g/ml final of purified mAb to CD4 (GK1.5) (ref. 21) or mAb against murine CD8 (53-6.7) (ref. 22) before adding chromium labelled BC or BCH4 cells. To determine the effect of anti-class I MHC antibodies on CTL killing, the chromium labelled target cells BC or BCH4 were incubated with 20 μ L of culture supernate of hybridoma that secretes a mAb that recognizes K^d and D^d of class I MHC (34-1-2S) (ref. 23) prior to the addition of effector cells.

Example 3

This Example describes the immunogenicity and protection by polynucleotide immunization by the intramuscular route.

To characterize the antibody responses following i.m. DNA administration, immune sera were analyzed for anti-RSV F IgG antibody titre by ELISA and for RSV-specific plaque reduction titre, respectively. All four plasmid constructs were found to be immunogenic. Sera obtained from mice immunized with pXL1-4 demonstrated significant anti-RSV F IgG titres and RSV-specific plaque reduction titres as compared to the placebo group (Table 1 below) ($P < 0.0061$ and < 0.0001 , respectively, Mann-Whitney Test). However, there is no significant difference in either anti-RSV F IgG titre or RSV-specific plaque reduction titre among mice immunized with either

pXL1, pXL2, pXL3 or pXL4.

To evaluate the protective ability of pXL1-4 against primary RSV infection of the lower respiratory tract, immunized mice were challenged intranasally with mouse-adapted RSV and viral lung titres post challenge were assessed. All four plasmid constructs were found to protect animals against RSV infection. A significant reduction in the viral lung titre was observed in mice immunized with pXL1-4 as compared to the placebo group ($P < 0.0001$, Mann-Whitney Test). However, varying degrees of protection were observed depending on the plasmid. In particular, pXL1 was more protective than pXL3 ($P = 0.00109$, Mann-Whitney Test), and pXL4 more than pXL3 ($P = 0.00125$), whereas only pXL2 induced complete protection. This conclusion was confirmed by another analysis with number of fully protected mice as end point (Fisher Exact Test). Constructs pXL1, pXL2 or pXL4 conferred a higher degree of protection than pXL3 ($P < 0.004$, Fisher Exact Test) which was not more effective than placebo. Only pXL2 conferred full protection in all immunized mice.

The above statistical analysis revealed that pXL1 conferred more significant protection than pXL3. The former expresses the truncated and secretory form and the latter the intact membrane anchored form of the RSV F protein. Furthermore, pXL4 was shown to be more protective than pXL3. The difference between these two constructs is the presence of the intron II sequence in pXL4. Construct pXL2 which expresses the secretory form of the RSV-F in the context of the intron II sequence was the only plasmid that confers complete protection in all immunized mice.

Example 4

This Example describes the influence of the route of administration of pXL2 on its immunogenicity and protective ability.

The i.m. and i.d. routes of DNA administration were compared for immunogenicity in terms of anti-RSV F antibody titres and RSV-specific plaque reduction titres. Analyses of the immune sera (Table 2 below) revealed that the i.d. route of DNA administration was as immunogenic as the i.m. route as judged by anti-RSV F IgG and IgG1 antibody responses as well as RSV-specific plaque reduction titres. However, only the i.m. route induced significant anti-RSV F IgG2a antibody responses, whereas the IgG2a isotype titre was negligible when the i.d. route was used. The i.m. and i.d. routes were also compared with respect to the induction of RSV-specific CTL. Significant RSV-specific CTL activity was detected in mice immunized intramuscularly. In contrast, the cellular response was significantly lower in mice inoculated intradermally (Table 3 below). In spite of these differences, protection against primary RSV infection of the lower respiratory tract was observed in both groups of mice immunized via either route (Table 4 below). The CTL induced by RSV-F DNA are classical CD8+ class I restricted CTL. The target cells, BCH4 fibroblasts express class I MHC only and do not express class II MHC. Further, prior incubation of BCH4 target cells with anti class-I MHC antibodies significantly blocked the lytic activity of RSV-F DNA induced CTL line. While anti-CD8 antibody could partially block lysis of BCH4 cells, antibody to CD4 molecule had no effect at all (Table 5 below). Lack of total blocking by mAb to CD8 could either be due to CTL being CD8 independent (meaning that even though they are CD8+ CTL, their TCR has enough affinity for class I MHC+peptide and it does not require CD8 interaction with the alpha 3 of class I MHC) or the amount of antibody used in these experiments was limiting. There was no detectable lysis of YAC-1 (NK sensitive target) cells (data not shown).

Example 5

This Example describes immunization studies in cotton rats using pXL2.

The immune response of cotton rats to DNA immunization was analyzed by the protocol shown in Table 6 below. On day -5, 40 cotton rats were randomly selected and divided into 8 groups of 5. Cotton rats in groups 1 and 7 were inoculated intramuscularly (i.m.) into the tiberlia arteria (TA) muscles bilaterally with cardiotoxin (1.0 μ M). On day -1, the cotton rats in group 8 were inoculated in the TA muscles with bupivacaine (0.25%). On day 0, several animals in each group were bled to determine levels of RSV-specific antibodies in the serum of the test animals prior to administration of vaccines. All of the animals were then inoculated i.m. or intradermally (i.d.) with 200 μ g of plasmid DNA, placebo (non-RSV-specific DNA), 100 median cotton rat infectious doses (CRID50; positive control) of RSV, or of formalin inactivated RSV prepared in Hep-2 tissue culture cells and adjuvanted in alum. Forty-four days later the cotton rats in groups 1 & 7 were reinoculated with cardiotoxin in the TA muscles. Four days later (48 days after priming with vaccine), the animals in group 8 were reinoculated with bupivacains in the TA muscle of the right leg. The next day, (seven weeks after priming with vaccine) all of the animals were bled and all, except those in the group given live RSV, were boosted with the same material and doses used on day 0. 29 days later, each cotton rat was bled and then challenged intranasally (i.n.) with 100 CRID50 RSV A2 grown in Hep-2 tissue culture cells. Four days after this virus challenge (day +88) all of the cotton rats were killed and their lungs removed. One lobe from each set of lungs was fixed in formalin and then processed for histologic evaluation of pulmonary histopathology. The remaining lobes of lung will be assessed for the presence

and levels of RSV. Each of the sera collected on days 0, 49 and 78 were tested for RSV-neutralizing activity, anti-RSV fusion activity and RSV-specific ELISA antibody.

The RSV neutralizing titres on day +49 and +78 are shown in Tables 7(a) below and 7(b) below respectively.

As can be seen from the results shown in Table 7(a), on day +49 the animals immunized with live RSV and DNA immunization had substantial RSV serum neutralizing titres. The animals immunized with formalin-inactivated RSV had a neutralizing titre equivalent to the placebo group on day +49 but following boosting titres by day +78 had reached 5.8 ($\log_{10}/0.05$). Boosting had no significant effect upon animals immunized with live RSV or by i.m. plasmid immunization.

RSV titres in nasal washes (upper respiratory tract) on day +82 are shown in Table 8 below. RSV titres in the lungs (lower respiratory tract) on day +82 are shown in Table 9 below. All of the vaccines provided protection against lung infection but under these conditions, only live virus provided total protection against upper respiratory tract infection.

The lungs from the cotton rats were examined histologically for pulmonary histopathology and the results are shown in Table 10 below. With the exception of lung sections obtained from Group 9 which were essentially free of inflammatory cells or evidence of inflammation, and those from Group 3, which exhibited the maximal pulmonary pathology seen in this study, all of the sections of lung obtained from the other groups looked familiar, i.e. scattered inflammatory cells were present in most fields, and there was some thickening of septae. These are evidence of mild inflammatory diseases. Large numbers of inflammatory cells and other evidence of inflammation were present in sections of lung from Group 3 (in which formalin-inactivated [FI] RSV vaccine was given prior to virus challenge). This result

indicated that immunization with plasmid DNA expressing the RSV F protein does not result in pulmonary histopathology different from the placebo, whereas FI-RSV caused more severe pathology.

5

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides certain novel vectors containing genes encoding an RSV F proteins, methods of immunization using such
10 vectors and methods of diagnosis using such vectors. Modifications are possible within the scope of this invention.

Table 1: Immunogenic and Protective Abilities of pXL1-4 Mice via the i.m. Route

Plasmid DNA Immunogen	No. Mice	Mean Anti-RSV F ELISA Titre(IgG) ^a (Log ₁ /100±SD)	Mean Plaque Reduction Titre ^a (Log ₁ ±SD)	Post RSV Challenge	
				Mean Virus Lung Titre# (pfu/g lung) (Log ₁₀ ±SD)	No. Fully Protected Mice ^{a,b}
pXL1	8	3.00±1.85	3.74±0.98	0.72±0.99	5
pXL2	9	5.78±1.72	4.82±0.51	0.00±0.00	9
pXL3	8	3.75±2.05	4.59±1.16	2.77±0.72	0
pXL4	9	5.44±1.13	5.18±0.43	0.66±1.00	6
Placebo ^{**}	12	0.58±2.89	0.18±0.62	3.92±0.27	0

^a These sets of data from sera obtained 1 week prior to the viral challenge

[#] Detection sensitivity of the assay was 10^{1.88} pfu/g lung.

^{a,b} The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 2. Immunogenicity of pXL2 in Mice*

Route	No. Mice	Mean Anti-RSV F ELISA Titre (Log ₂ /100 + SD)			Mean Plaque Reduction Titre (Log ₄ ± SD)
		IgG	IgG1	IgG2a	
i.m.	8	7.63±0.92	4.25±1.91	4.38±1.92	4.18±0.88
i.d.	7	7.00±1.00	5.00±1.00	0.14±0.38	3.65±0.59
Placebo (i.m.)	9	0.50±0.51	0.00±0.00	0.00±0.00	0.18±0.50

* These sets of data are from sera obtained 1 week prior to the viral challenge.

Table 3. Induction of RSV-specific CTL Following DNA Immunization*

Route	E:T Ratio	% Specific Lysis	
		BC	BCH4
i.m.	200:1	23.3	100.6
	100:1	17.0	62.4
	50:1	19.9	64.1
	25:1	22.3	46.4
i.d.	100:1	20.9	26.1
	50:1	21.7	19.1
	25:1	7.1	7.0
	12.5:1	2.8	2.3

* These set of data were obtained from immunized mice immediately prior to RSV challenge.

Table 4. Immunoprotective Ability of pXL2 in Mice

Route	No. Mice	Post RSV Challenge	
		Mean Virus Lung Titre* (pfu/g lung)	No. Fully Protected Mice#
i.m.	8	0.00±0.00	8
i.d.	7	0.43±1.13	6
Placbo (i.m.)	9	4.30±0.22	0

* Detection sensitivity of the assay was $10^{1.69}$ pfu/g lung.

The term, fully protected mice, refers to animals with no detectable RSV in lungs post challenge.

Table 5. RSV specific CTL included by i.m. DNA immunization are class I restricted CTL

E:T Ratio	BCH4	BCH4+anti-CD4	BCH4+anti-CD8	BCI4+anti-class I MHC
100:1	52.03	54.3	39.4	8.6
50:1	44.4	47.2	27.4	6.2
25:1	28.6	26.3	14.8	1
12.5:1	18.2	15	8	-2.7

Table 6

Group	Antigen	RSV-specific dose	Inoc. route	Pretreatment/Adjuvant	Day 0	Day 49	Day 78	Day 88
1	Placebo	0	I.M.	Cardiotoxin	Prebleed, several cotton rats per group; prime all animals	Bleed all animals; boost all except those in group 2	Challenge with RSV A2 I.N. after bleeding all	Harv. animals and do histologic evaluation, pulmonary virus titers, antibodies
2	Live RSV	100 CRID50	I.N.	None				
3	FI-RSV		I.M.	Alum				
5	pXL2	200 µg	I.M.	None				
6	pXL2	200 µg	I.D.	None				
7	pXL2	200 µg	I.M.	Cardiotoxin				
8	pXL2	200 µg	I.M.	Bupivacaine				

Table 7(a). RSV Serum Neutralizing Titers on Day 49

Group	Antigen	RSV- specific dose	Inoc. route	Nt. antibody titer ($\log_2/0.05$ ml) in CR no.				Mean titer $\log_2/$ 0.05	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4	3	2	2	2.75	1.0
2	Live RSV	100 CRID50	I.N.	9	9	9	9	9	0.0
3	FI-RSV		I.M.	0	4	2	2	2.0	1.6
5	pXL2	200 μ g	I.M.	9	8	8	7	8.0	0.8
6	pXL2	200 μ g	I.D.	5	2	5	5	4.3	1.5
7	pXL2	200 μ g	I.M.	8	8	9	9	8.5	0.6
8	pXL2	200 μ g	I.M.	8	9	6	6	7.3	1.5

Table 7(b). RSV Serum Neutralizing Titers on Day 78

Group	Antigen	RSV specific dose	Inoc. route	Nt. antibody titer ($\log_2/0.05$ ml) in CR no.					Mean titer $\log_2/0.05$	Stand. Dev.
				1	2	3	4	5		
1	Placebo	0	I.M.	3	2	4	Died		3.0	1.0
2	Live RSV	100 CRD50	I.N.	8	9	8	9		8.5	0.6
3	FI-RSV		I.M.	8	4	6	5		5.8	1.7
5	pXL2	200 μ g	I.M.	7	8	8	8		7.8	0.5
6	pXL2	200 μ g	I.D.	8	6	6	Died		6.7	1.2
7	pXL2	200 μ g	I.M.	8	9	9	8		8.7	0.6
8	pXL2	200 μ g	I.M.	8	7	9	9		8.3	1.0

Table 8. RSV Titers in Nasal Washes on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer ($\log_{10}/0.05$ ml) in cotton rat no.				Mean titer $\log_{10}/0.05$	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	3.4	3.3	3.3	Died	3.3	0.1
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV		I.M.	0	0	2.8	0	0.7	1.4
5	pXL2	200 μ g	I.M.	3.3	2.3	3.3	2.3	2.8	0.6
6	pXL2	200 μ g	I.D.	N.D.	N.D.	N.D.	Died	N.D.	N.D.
7	pXL2	200 μ g	I.M.	2.3	0	0	3.2	1.4	1.6
8	pXL2	200 μ g	I.M.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. = non-determined

Table 9. Titers in Lungs on Day 82

Group	Antigen	RSV-specific dose	Inoc. route	RSV titer (\log_{10} /g lung) in cotton rat no.				Mean titer $\log_{10}/0.05$	Stand. Dev.
				1	2	3	4		
1	Placebo	0	I.M.	4.7	4.2	3.7	Died	4.2	0.5
2	Live RSV	100 CRID50	I.N.	0	0	0	0	0.0	0.0
3	FI-RSV	10^5 PFU	I.M.	0	0	0	0	0.0	0.0
5	pXL2	200 μ g	I.M.	0	2.2	0	0	0.6	1.1
6	pXL2	200 μ g	I.D.	0	2.2	2.7	3.2	2.0	N.D.
7	pXL2	200 μ g	I.M.	0	0	0	0	0.0	0.0
8	pXL2	200 μ g	I.M.	0	0	0	0	0.0	N.D.

N.D. = non-determined

Table 10. Summary of Histopathology Results Seen in Sections of Cotton Rat Lung.

Group	Treatment	Major Observations & Comments
1.	Placebo + RSV	Scattered individual and groups of macrophages and polymorphonuclear neutrophils (PMN) in all fields. Overt thickening of septae. Occasional pyknotic cells seen. Overall: mild to moderate inflammation.
2.	Live RSV	Isolated macrophages seen in most fields. Scattered PMN. Overall: minimal inflammation
3.	FI-RSV + RSV	Virtually every field contains numerous mononuclear cells & PMN. Pyknotic cells and debris common. Thickened septae. Evidence of exacerbated disease.
5.	Plasmid + RSV	Isolated macrophages seen in most fields. Occasional PMN seen. Very similar to live virus group.
6.	Plasmid i.d. + RSV	Isolated macrophages seen in most fields. Occasional PMN seen.
7.	Plasmid + CT + RSV	Isolated mononuclear cells and PMN seen in most fields.
8.	Plasmid + Biv + RSV	Scattered mononuclear cells and PMN seen in most fields.
9.	Normal CR Lung	Few leukocytes evidence. Airy, open appearance. Thin septae.

CT = carditoxin

Biv = bupivacaine

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CLAIMS

What we claim is:

1. A vector, comprising:
 - a first nucleotide sequence encoding an RSV F protein or a protein capable of inducing antibodies that specifically react with RSV F protein;
 - a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein, and
 - a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from said vector in a host.
2. The vector of claim 1 wherein said first nucleotide sequence encodes a full-length RSV F protein.
3. The vector of claim 1 wherein said first nucleotide sequence encodes a RSV F protein from which the transmembrane region is absent.
4. The vector of claim 1 wherein said first nucleotide sequence encodes a full-length RSV F protein and contains a translational stop codon immediately upstream of the start of the transmembrane coding region to prevent translation of the transmembrane coding region.
5. The vector of claim 1 wherein said promoter sequence is an immediate early cytomegalovirus promoter.
6. The vector of claim 1 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all RNA transcribed encodes an RSV F protein.
7. The vector of claim 6 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.
8. The vector of claim 7 wherein said second nucleotide sequence is that of rabbit β -globin intron II.
9. The vector of claim 1 which is pXL2 as shown in Figure 5.

10. The vector of claim 1 which is pXL4 as shown in Figure 7.
11. A vector, comprising:
 - a first nucleotide sequence encoding an RSV F protein or a protein capable of generating antibodies that specifically react with RSV F protein, and
 - a cytomegalovirus promoter operatively coupled to said first nucleotide sequence for expression of said RSV F protein.
12. A vector of claim 11 wherein said first nucleotide sequence encodes a full-length RSV F protein.
13. A vector of claim 11 wherein said first nucleotide sequence encodes a RSV F protein from which the transmembrane region is absent.
14. The vector of claim 11 wherein said first nucleotide sequence encodes a full-length RSV F protein and contains a translational stop codon immediately upstream of the start of the transmembrane coding region to prevent translation of the transmembrane coding region.
15. The vector of claim 11 further comprising a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from said vector in a host.
16. The vector of claim 15 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes an RSV F protein.
17. The vector of claim 16 wherein said second nucleotide sequence is located between said first nucleotide sequence and said cytomegalovirus promoter.
18. The vector of claim 17 wherein said second nucleotide sequence is that of rabbit β -globin intron II.
19. The vector of claim 11 which is pXL1 as shown in Figure 4.
20. The vector of claim 11 which is pXL3 as shown in

Figure 6.

21. A method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to said host an effective amount of a vector comprising a first nucleotide sequence encoding an RSV F protein or a protein capable of inducing antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host.
22. The method of claim 21, wherein the first nucleotide sequence encodes a full-length RSV F protein and contains a translational stop codon immediately upstream of the start of the transmembrane coding region to prevent translation of the transmembrane coding region.
23. The method of claim 21 wherein said host is a human.
24. The method of claim 23 wherein said promoter sequence is an immediate early cytomegalovirus promoter.
25. The method of claim 21 wherein said vector is pXL1 as shown in Figure 4.
26. The method of claim 21 wherein said vector is pXL2 as shown in Figure 5.
27. A method of immunizing a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises administering to said host an effective amount of a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein, or an RSV protein lacking a transmembrane region, a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein, and a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from said vector in said host.

28. The method of claim 27 wherein said first nucleotide sequence encodes a full-length RSV F protein.

29. The method of claim 27 wherein said first nucleotide sequence encodes an RSV F protein from which the transmembrane region is absent.

30. The method of claim 27 wherein said first nucleotide sequence encodes a full-length RSV F protein and contains a translational stop codon immediately upstream of the start of the transmembrane coding region to prevent translation of the transmembrane coding region.

31. The method of claim 27 wherein said promoter sequence is an immediate early cytomegalovirus promoter.

32. The method of claim 31 wherein said second nucleotide sequence comprises a pair of splice sites to prevent aberrant mRNA splicing, whereby substantially all transcribed mRNA encodes an RSV F protein.

33. The method of claim 32 wherein said second nucleotide sequence is located between said first nucleotide sequence and said promoter sequence.

34. The method of claim 33 wherein said second nucleotide sequence is that of rabbit β -globulin intron II.

35. The method of claim 27 wherein said vector is pXL1 as shown in Figure 4.

36. The method of claim 27 wherein said vector is pXL3 as shown in Figure 6.

37. A method of using a gene encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein or an RSV F protein lacking a transmembrane region to produce an immune response in a host, which comprises:

- isolating said gene;
- operatively linking said gene to at least one control sequence to produce a vector, said control sequence directing expression of said RSV F protein when said vector is introduced into a host to produce an

immune response to said RSV F protein; and
introducing said vector into the host.

38. The method of claim 37 wherein said gene encoding an RSV F protein encodes an RSV F protein lacking the transmembrane region.

39. The method of claim 38 wherein said at least one control sequence comprises the immediate early cytomegalovirus promoter.

40. The method of claim 39 including the step of:
operatively linking said gene to an immunoprotective enhancing sequence to produce an enhanced immunoprotection to said RSV F protein in said host.

41. The method of claim 40 wherein said immunoprotective enhancing sequence is introduced into said vector between said control sequence and said gene.

42. The method of claim 41 wherein said immunoprotection enhancing sequence comprises a pair of splice sites to prevent aberrant mRNA splicing whereby substantially cell transcribed mRNA encodes an RSV F protein.

43. The method of claim 42 wherein said immunoprotection enhancing sequence is that of rabbit β -globin intron II.

44. The method of claim 37 wherein said gene is contained within a plasmid selected from the group consisting of pXL1, pXL2 and pXL4.

45. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein or a protein capable of generating antibodies that specifically react with RSV F protein;

operatively linking said first nucleotide sequence to at least one control sequence to produce a vector; the control sequence directing expression of said RSV F protein when introduced into a host to produce an immune response to said RSV F protein;

operatively linking said first nucleotide sequence

to a second nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed in vivo from the vector in a host, and

formulating said vector as a vaccine for in vivo administration.

46. The method of claim 45 wherein said vector is selected from the group consisting of pXL1, pXL2 and pXL4.

47. A vaccine produced by the method of claim 45.

48. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first nucleotide sequence encoding an RSV F protein from which the transmembrane region is absent;

operatively linking said first nucleotide sequence to at least one control sequence to produce a vector, the control sequence directing expression of said RSV F protein when introduced into a host to produce an immune response to said RSV F protein; and

formulating said vector as a vaccine for in vivo administration.

49. The method of claim 48 wherein said vector is selected from group consisting of pXL1 and pXL2.

50. A vaccine produced by the method of claim 48.

51. A method of determining the presence of a respiratory syncytial virus (RSV) F protein in a sample, comprising the steps of:

(a) immunizing a host with a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein, or a RSV F protein lacking a transmembrane region, and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific

for the RSV F protein;

(b) isolating the RSV F protein specific antibodies;

(c) contacting the sample with the isolated antibodies to produce complexes comprising any RSV F protein present in the sample and said isolated RSV F protein-specific antibodies; and

(d) determining production of the complexes.

52. The method of claim 51 wherein said vector is selected from the group consisting of pXL1, pXL2, pXL3 and pXL4.

53. A diagnostic kit for detecting the presence of an RSV F protein in a sample, comprising:

(a) a vector comprising a first nucleotide sequence encoding an RSV F protein, a protein capable of generating antibodies that specifically react with RSV F protein, or a RSV F protein lacking a transmembrane region, and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in a host immunized therewith to produce antibodies specific for the RSV F protein;

(b) isolation means to isolate said RSV F protein-specific antibodies;

(c) contacting means to contact the isolated RSV F specific antibodies with the sample to produce a complex comprising any RSV F protein present in the sample and RSV F protein specific antibodies, and

(d) identifying to determine production of the complex.

54. The diagnostic kit of claim 53 wherein said vector is selected from the group consisting of pXL1, pXL2, pXL3 and pXL4.

55. A method for producing antibodies specific for an F protein of RSV comprising:

(a) immunizing a host with an effective amount of

a vector comprising a first nucleotide sequence encoding an RSV F protein lacking a transmembrane region and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein in said host to produce antibodies specific for the F protein; and

(b) isolating the antibodies from the host.

56. A method of producing monoclonal antibodies specific for an F protein of RSV comprising the steps of:

(a) constructing a vector comprising a first nucleotide sequence encoding an RSV F protein and a promoter sequence operatively coupled to said first nucleotide sequence for expression of said RSV F protein; and, optionally,

a second nucleotide sequence located adjacent said first nucleotide sequence to enhance the immunoprotective ability of said RSV F protein when expressed *in vivo* from said vector in a host.

(b) administering the vector to at least one mouse to produce at least one immunized mouse;

(c) removing B-lymphocytes from the at least one immunized mouse;

(d) fusing the B-lymphocytes from the at least one immunized mouse with myeloma cells, thereby producing hybridomas;

(e) cloning the hybridomas;

(f) selecting clones which produce anti-F protein antibody;

(g) culturing the anti-F protein antibody-producing clones; and

(h) isolating anti-F protein monoclonal antibodies from the cultures.

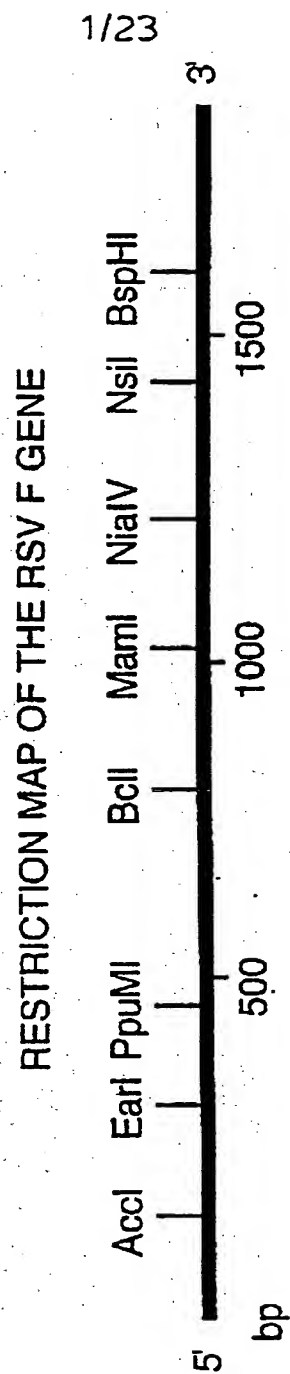


FIG.1

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FIG.2

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA ALA VAL THR PHE
 ATGGAGTTGCCAATCCTCAAGCAAAATGCAATTAACCAATCCTCGCTGCAGTCACATTT
 TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTAGGAGCGACGTCAGTGTAA
 10 20 30 40 50 60
 CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
 TGCTTTGCTTCTAGTCAAAACATCACTGAGAAATTTTATCAATCAACATGCAGTGCAGTT
 ACGAAACGAAGATCAGTTTGTAGTGAATCTTAAATAGTTAGTTGTACGTACGTCACGTCAA
 70 80 90 100 110 120
 SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
 AGCAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATAGTGTATTAACATATAGAA
 TCGTTTCCGATAGAAATCAGGAGATCTTGACCAACCATATGATCACAATATGATATCTTT
 130 140 150 160 170 180
 LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
 TTAAGTAATATCAAGGAAATAAGTGTAAATGGAACAGATGCTAAGGTAAATGTATGAA
 AATTCAATTAGTTTCCTTTTATTCACATTACCTTGCTACGATTCCATTTTAACTACTTT
 190 200 210 220 230 240
 GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU MET GLN SER THR
 CAAGAATTAGATAAATATAAAATGCTGTAACAGAAATGCGAGTTGCTCATGCAAGCACA
 GTTCTTAATCTATTTATATTTTACGACATTGTCTTAACGTCAACGAGTACGTTTCGTGT
 250 260 270 280 290 300
 PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
 CCAGCAGCAACAATCGAGCCAGAGAACTACCAAGGTTTATGAAATATACACTCAAC
 GGTCGTCGTTTGTAGCTCGGTCTTCTTGTATGGTTCCAAATACTTAATATGTGAGTTG
 310 320 330 340 350 360

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FIG.3

NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

5' MET GLU LEU PRO ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA VAL THR PHE
 ATGGAGTTGCCAATCCTCAAGCAAAATGCAATTAACCAATCCTCGCTGCAGTCACATTT
 TACCTCAACGGTTAGGAGTTTCGTTTACGTTAATGGTGTAGGAGCGACGTCAGTGTAA
 10 20 30 40 50 60
 CYS PHE ALA SER SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL
 TGCTTTGCTTCTAGTCAAAACATCACTGAGAAATTTTATCAATCAACATGCAGTGCAGTT
 ACGAAACGAAGATCAGTTTGTAGTGTGACTTCTTAAATAAGTTAGTTGTACGTACGTCAAC
 70 80 90 100 110 120
 SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU
 AGCAAGGCTATCTTAGTGCTCTAAGAACTGGTTGGTATACTAGTGTATAACTATAGAA
 TCGTTCCGATAGAAATCAGGAGATTTCTTGACCAACCATATGATCAATAATGATATCTTT
 130 140 150 160 170 180
 LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU MET LYS
 TTAAGTAATATCAAGGAAAATAAGTGTAAATGGAACAGATGCTAAGGTAAATTTGATGAAA
 AATTCAATTATAGTTCTTTTATTACATTTACCTTGCTACGATTCCATTTTAACTACTTT
 190 200 210 220 230 240
 GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU GLN LEU MET GLN SER THR
 CAAGAAATTAGATAAATAAATAATGCTGTAAACAGAAATTGCAGTTGCTCATGCAAGCACA
 GTTCTTAAATCTATTTATATTTTACGACATTTGTCTTAAACGTCAACGAGTACGTTTCGTGT
 250 260 270 280 290 300
 PRO ALA ALA ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN
 CCAGCAGCAACAATCGAGCCAGAGAGAACTACCAAGGTTTATGAATTATACACTCAAC
 GGTCGTCGTTTGTAGTCGGTCTTCTCTTGTATGGTTCCAAATACTTAATATGTGAGTTG
 310 320 330 340 350 360

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FIG.3 CONT.

F2-F1CLEAVAGE SITE
 ASN THR LYS LYS THR ASN VAL THR LEU SER LYS LYS ARG LYS ARG ARG PHE LEU GLY PHE
 AATACCAAAACCAATGTAACATTAAGCAAGAAAGGAAA8AAGATTTCCTGGTTT
 TTATGGTTTTCCTGGTTACATTGTAATTTCGTTCTTTTCCTTTCTTAAAGAACCAAAA
 370 380 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY ILE ALA VAL SER LYS VAL LEU HIS LEU
 TTGTTAGGTGTTGGATCTGCAATCGCCAGTGGCATGCTGTATCTAAGGTCCTGCACCTTA
 AACAAATCCACAACCTAGACGTTAGCGGTACCGTACCGTAACGACATAGATTCCAGGACGTGAAT
 430 440 450 460 470 480

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU LEU SER THR ASN LYS ALA VAL VAL SER
 GAAGGAGAAGTGAACAAGATCAAAAGTGTCTTACTATCCACAACAAGCCGTAGTCAGC
 CTTCCCTCTTCACCTTGTCTTAGTTTTCACGAGATGATAGGTGTTTGTTCGGCATCAGTCG
 490 500 510 520 530 540

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP
 TTATCAATGGAGTTAGTGTCTTAACCAGCAAGTGTAGACCTCAAAAACATATATAGAT
 AATAGTTTACCTCAATCACAGAAATTGTTGTTTCACAACTCTGGAGTTTGTGATATATCTA
 550 560 570 580 590 600

LYS GLN LEU LEU PRO ILE VAL ASN LYS GLN SER CYS ARG ILE SER ASN ILE GLU THR VAL
 AAACAATTGTTACCTATTGTGAATAAGCAAGCAAGCTGCAGAAATATCAATATAGAAACTGTG
 TTTTGTAAACAATGGATAACACTTATTTCGTTTCGACGCTTATATAGTTTATATCTTTTGACAC
 610 620 630 640 650 660

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL ASN
 ATAGAGTTCACAACAAGAACACACAGACTACTAGAGATTACCAGGGAATTTAGTGTTAAT
 TATCTCAAGGTGTTTTCCTTGTGTTGTCTGATGATCTCTAATGTTCCCTTAAATCACAATTA
 670 680 690 700 710 720

ALA GLY VAL THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU LEU SER LEU
 GCAGGTGTAACCTACACCTGTAAGCATTACATGTTAACTAATAGTGAATTAATGTCATTA
 CGTCCACATTGATGTGGACATTCTGTAATGTACAATTTGATTATCATCTTAATAACAGTAAT
 730 740 750 760 770 780

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FIG.3 CONT.

ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LYS LEU MET SER ASN ASN VAL GLN ILE
 ATCAATGATATGCCCTATAACAATGATCAGAAAAGTTAATGTCCAAACAATGTTCAAATA
 TAGTTACTATACGGGATATTGTTTACTAGTCTTTTCAATTACAGGTTGTTACAAAGTTTAT
 790 800 810 820 830 840
 VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL
 GTTAGACAGCAAAAGTTACTCTATCATGTCCATAATAAAGAGGAAAGTCTTAGCATATGTA
 CAATCTGTCGTTTCAATGAGATAGTACAGGTATATTTCCTCCTTCAGAAATCGTATACAT
 850 860 870 880 890 900
 VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO
 GTACAATTACCACTATATGGTGTGATAGATACACCTTGTGGAAATACACACATCCCT
 CATGTTAATGGTGATATACCACACTATCTATGTGGAACAACCTTTAATGTGTGTAGGGGA
 910 920 930 940 950 960
 LEU CYS THR THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY
 CTATGTACAACCAACACAAAGAGGGTCAACATCTGTTTAAACAAGAACTGACAGAGGA
 GATACATGTTGGTTGTGTTTCTTCCAGTTGTAGACAAATGTTCTTGACTGTCTCCT
 970 980 990 1000 1010 1020
 TRP TYR CYS ASP ASN ALA GLY SER VAL SER PHE PHE PRO GLN ALA GLU THR CYS LYS VAL
 TGGTACTGTGACAATGCAGGATCAGTATCTTCTTCCCAAGCTGAAACATGTAAAGTT
 ACCATGACACTGTTACGTCCTAGTCATAGAAAGGGTGTTCGACTTTGTACATTTCAA
 1030 1040 1050 1060 1070 1080
 GLN SER ASN ARG VAL PHE CYS ASP THR MET ASN SER LEU THR LEU PRO SER GLU VAL ASN
 CAATCGAATCGAGTATTTGTGACACAATGAACAGTTTAACATTAACCAAGTGAAGTAAT
 GTTAGCTTAGCTCATATAAACAACACTGTGTACTTGTCAAATGTAAATGGTTCACCTTCATTTA
 1090 1100 1110 1120 1130 1140
 LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS LYS ILE MET THR SER LYS THR
 CTCTGCAATGTTGACATATTCAATCCCAATATGATGTGTAATAATATGACTTCAAAAACA
 GAGACGTTACAACACTGTATAAGTTAGGGTTTATACTAACAATTTTAATACTGAAGTTTGT
 1150 1160 1170 1180 1190 1200

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FIG.3 CONT.

ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR
GATGTAAGCAGCTCCGTTATACATCTTAGGAGCCATTGTGTCATGCTATGGCAAAACT
CTACATTCTGTCGAGGCAATAGTGTAGAGATCCTCGGTAACACAGTACGATACCGTTTGA
1210 1220 1230 1240 1250 1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE ILE LYS THR PHE SER ASN GLY CYS ASP
AAATGTACAGCATCCAATAAATAATCGTGAATCATAAAGACATTTTCTAAACGGGTGTGAT
TTTACATGTCGTAGGTTATTTTGTAGCACCTTAGTATTTCTGTAAAGATTGCCCACTA
1270 1280 1290 1300 1310 1320

TYR VAL SER ASN LYS GLY VAL ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN
TATGTATCAATAAAGGGGTGACACTGTGTCTGTAGGTAACACATTTATATTATGTAAT
ATACATAGTTTATTTCCCACTGTGACACAGACATCCATTGTGTAAATAATACATTTA
1330 1340 1350 1360 1370 1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO
AAGCAAGAAGGCAAAAGTCTCTATGTAAAGGTGAACCAATAAJAAATTTCTATGACCCA
TTTCGTTCTTCCGTTTTCAGAGATACATTTTCCACTTGGTTATTTAAAGATACTGGGT
1390 1400 1410 1420 1430 1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN
TTAGTATTCCCTCTGATGAATTGATGCAATCAATATCTCAAGTCAATGAGAAGATTAAAC
AATCATAAGGGGAGACTACTTAAACTACGTAGTTATAGAGTTCAGTTACTCTTCTAATTG
1450 1460 1470 1480 1490 1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS
CAGAGTTTAGCATTTTATTCGTAAATCCGATGAATTATTACATAATGTAATGCTGGTAA
GTCTCAAATCGTAAATAAGCATTTAGGCTACTTAATAATGTATTACATTTACGACCATTT
1510 1520 1530 1540 1550 1560

SER THR THR ASN ILE MET Thr Stop Stop Bam HI
TCAACCACAATAATCATGACTTGATTAATGAGGATCC
AGTIGGTGTTTATAGTACTGAACCTATTACTCTAGG
1570

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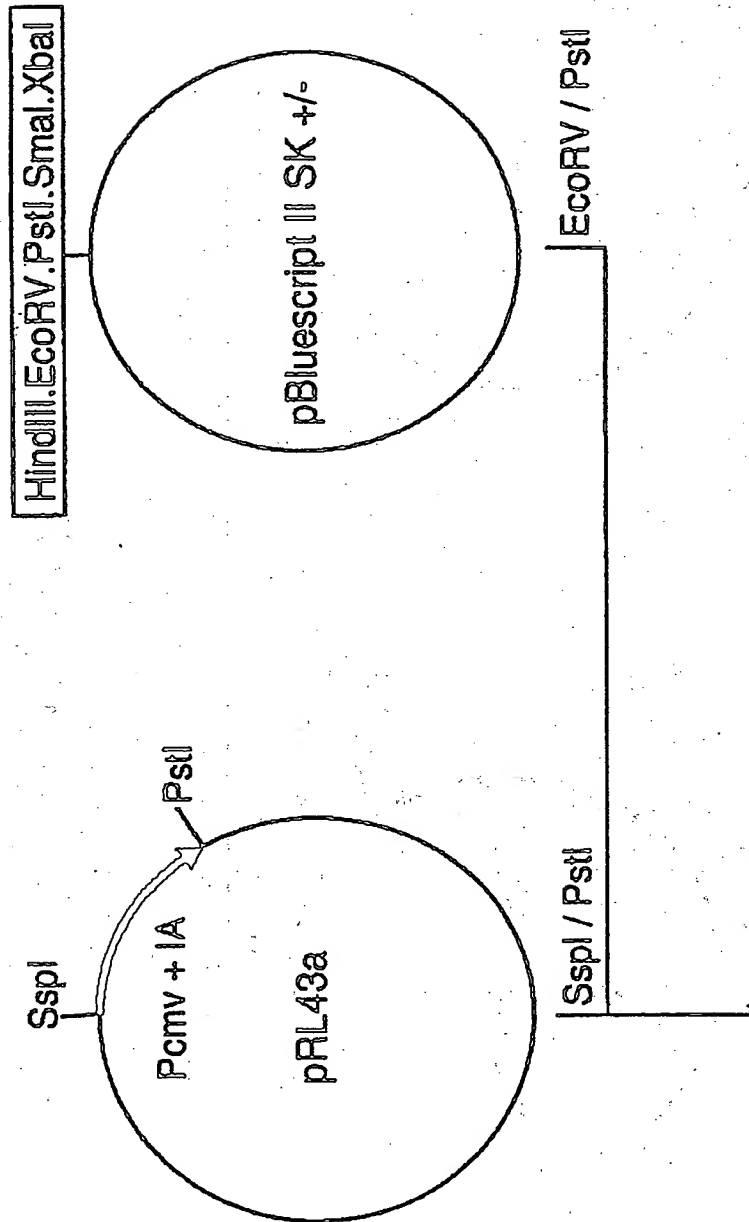


FIG.4A

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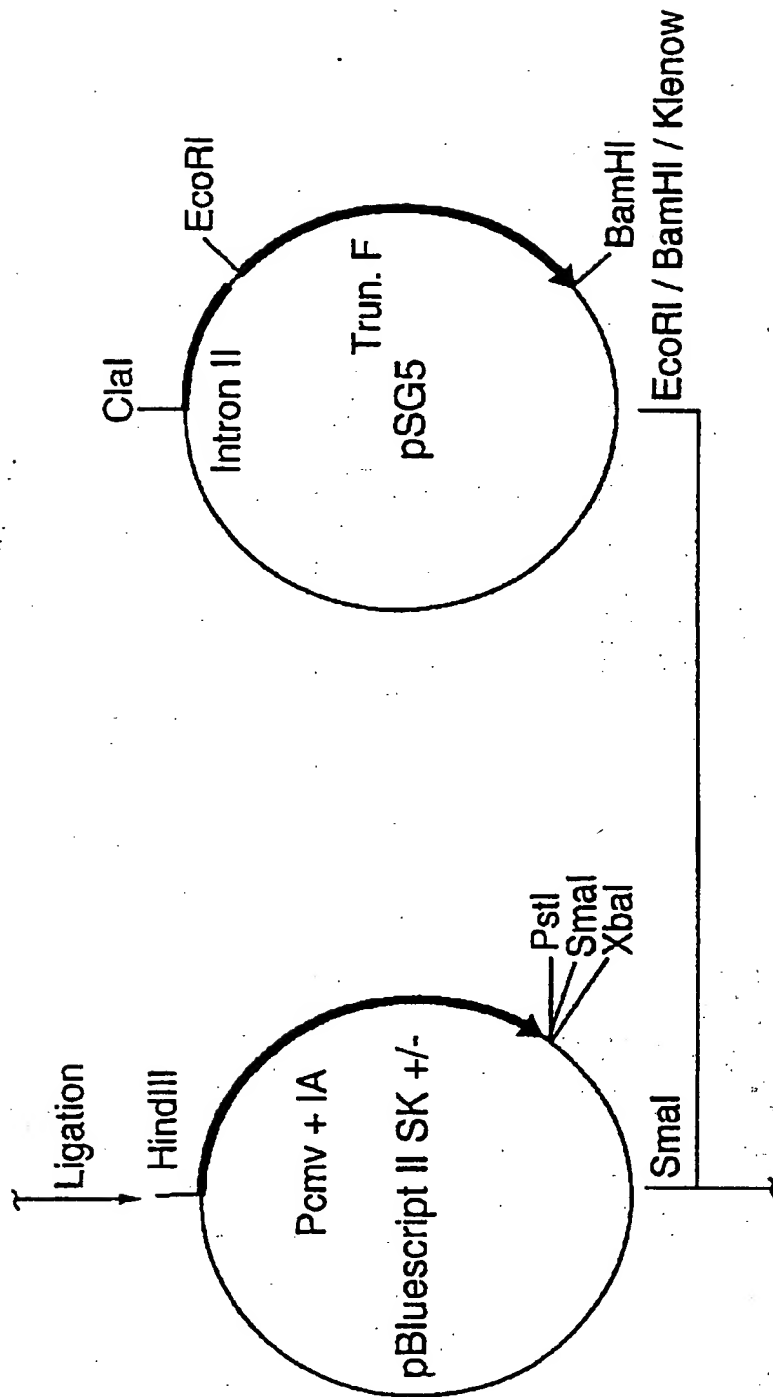


FIG.4B

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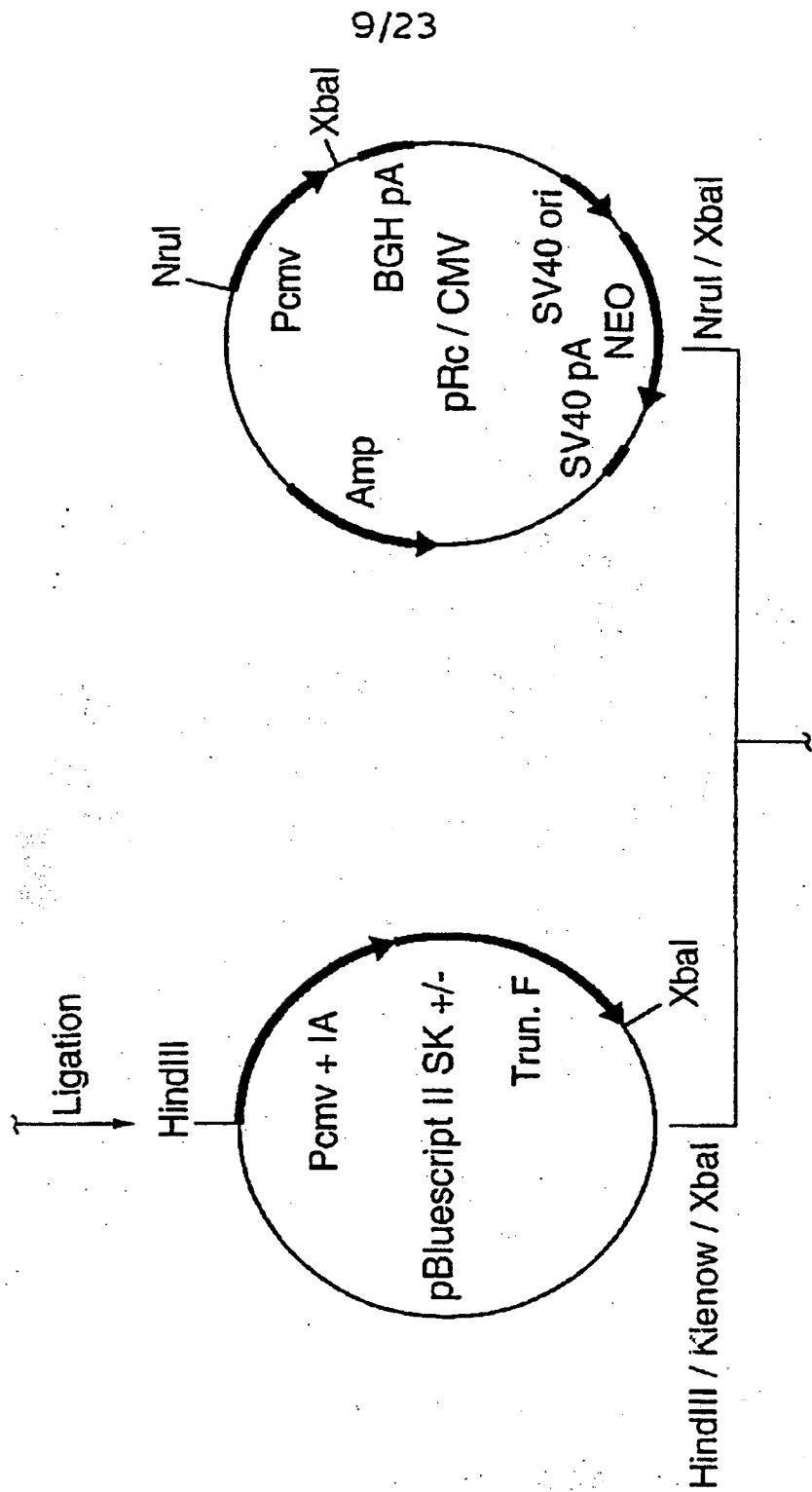


FIG.4C

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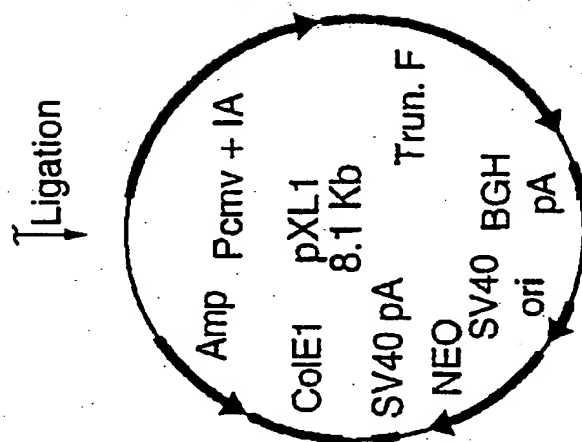


FIG.4D

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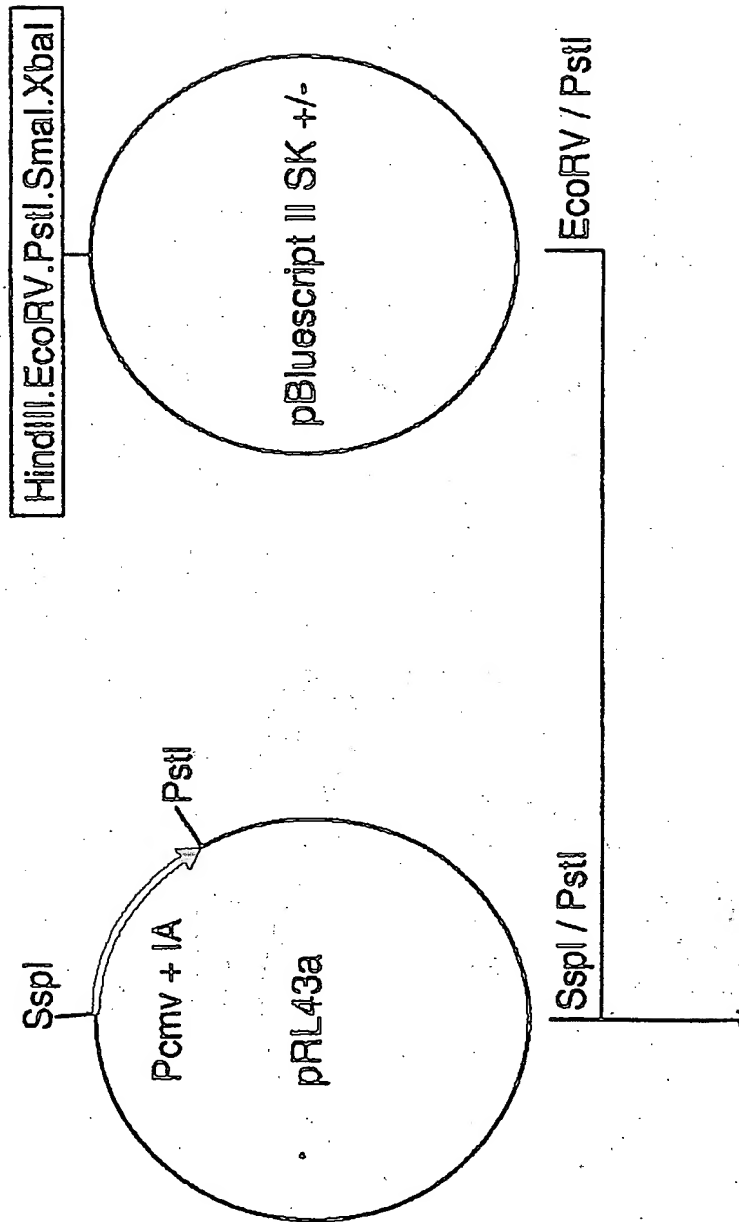


FIG.5A

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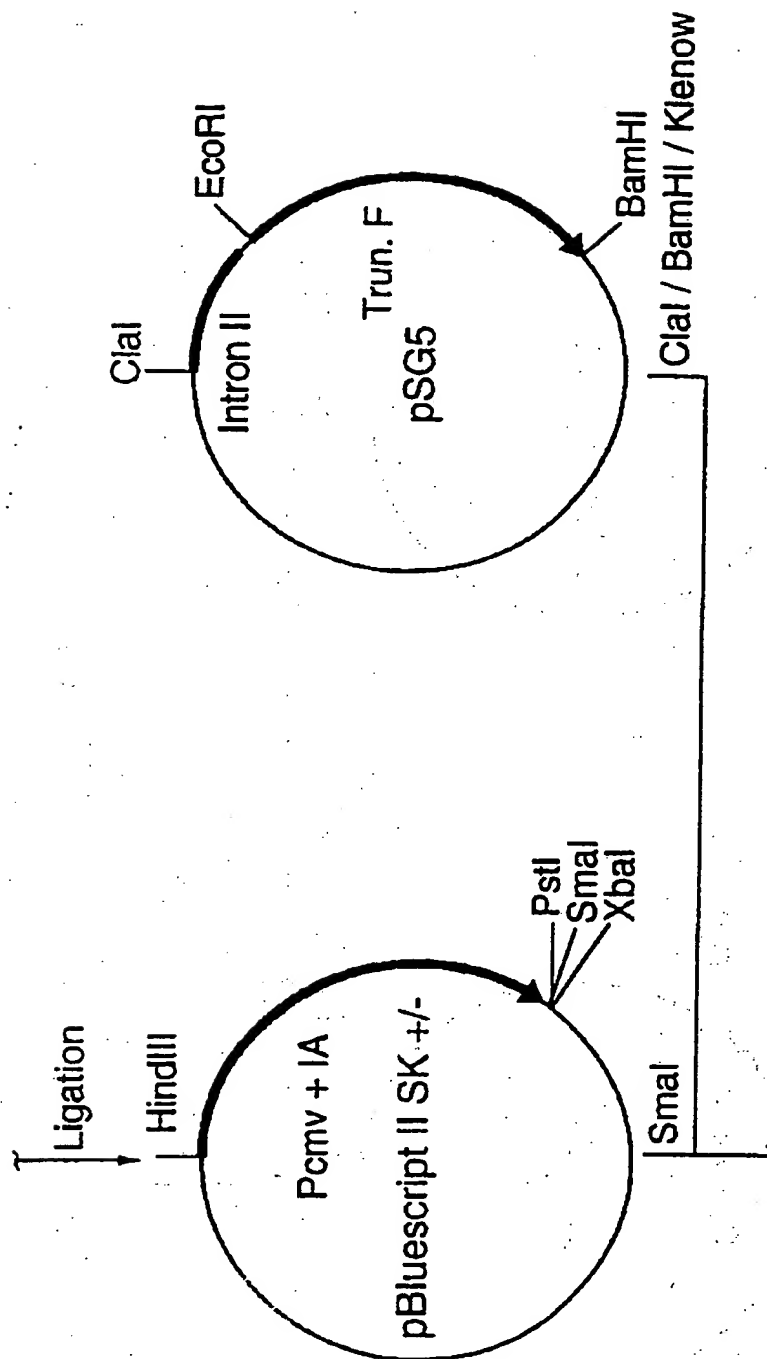


FIG.5B

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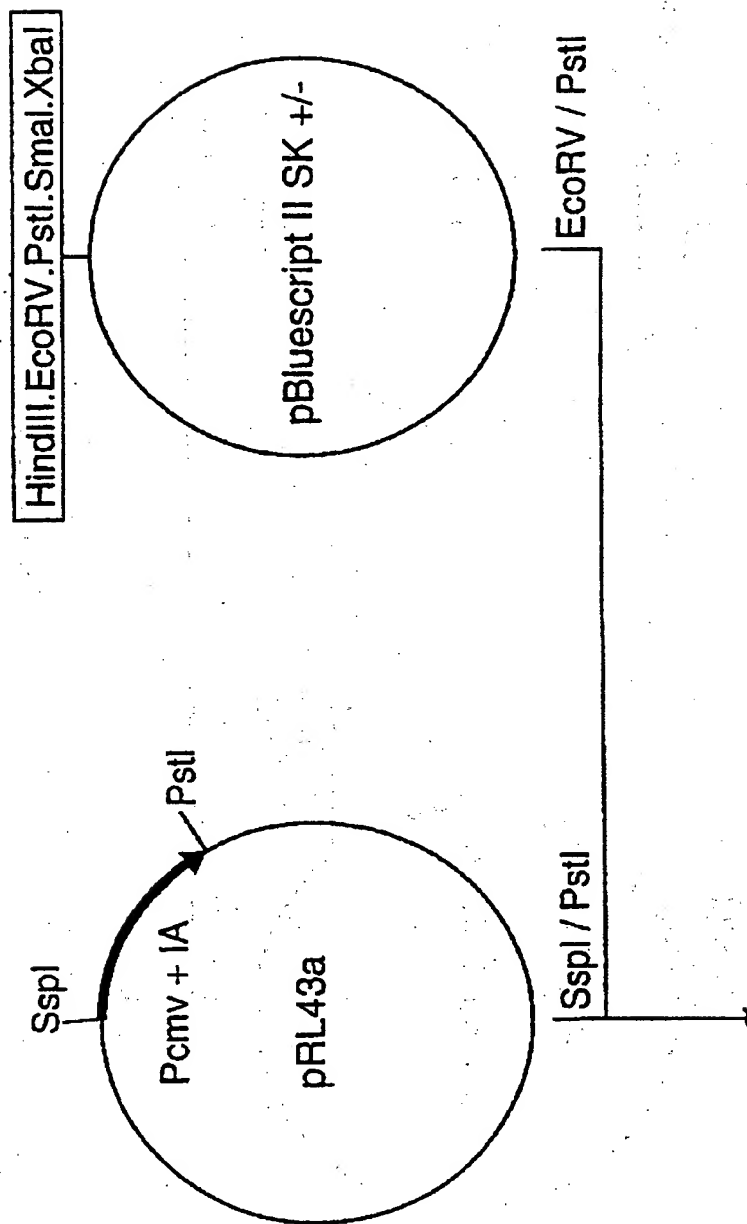


FIG.6A

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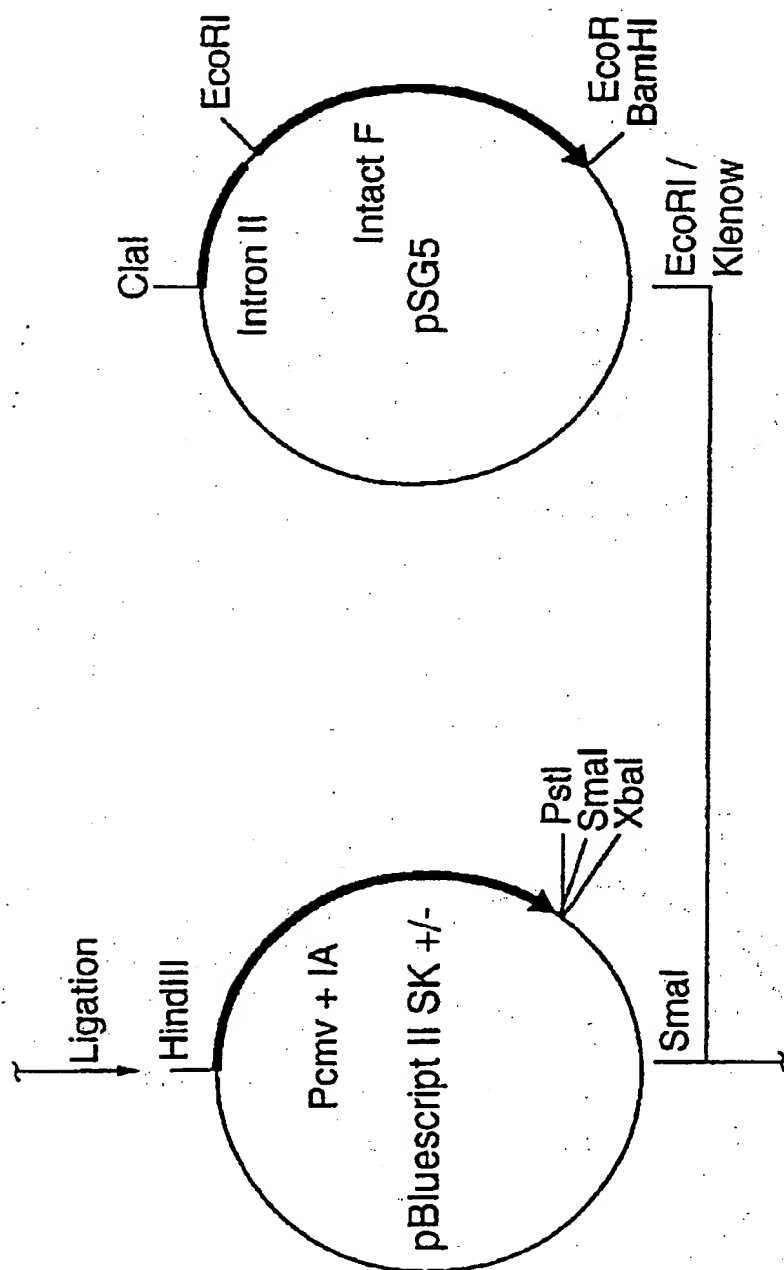


FIG.6B

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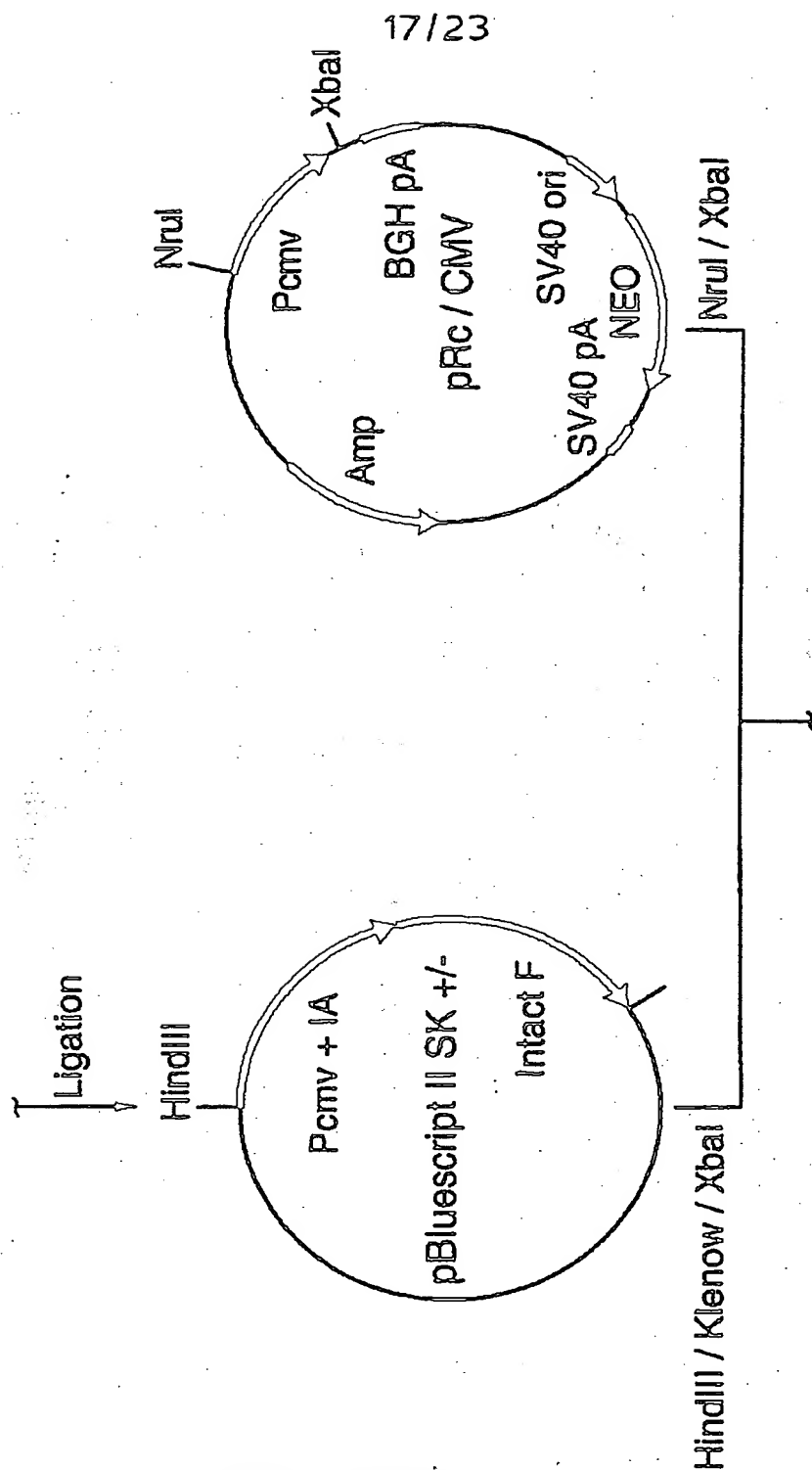


FIG.6C

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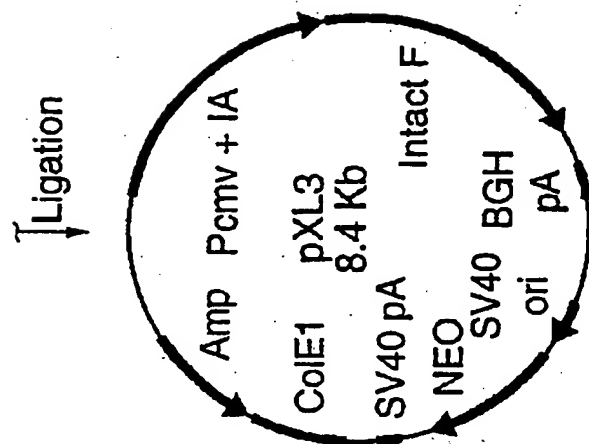


FIG.6D

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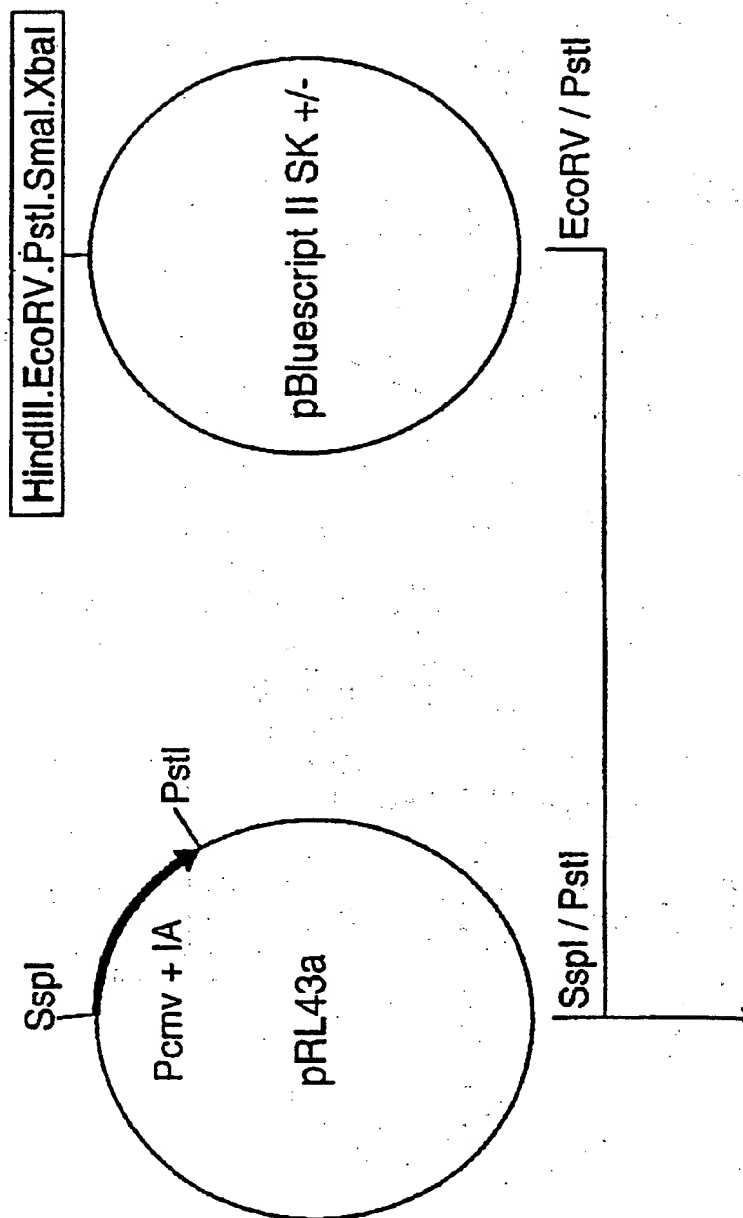


FIG.7A

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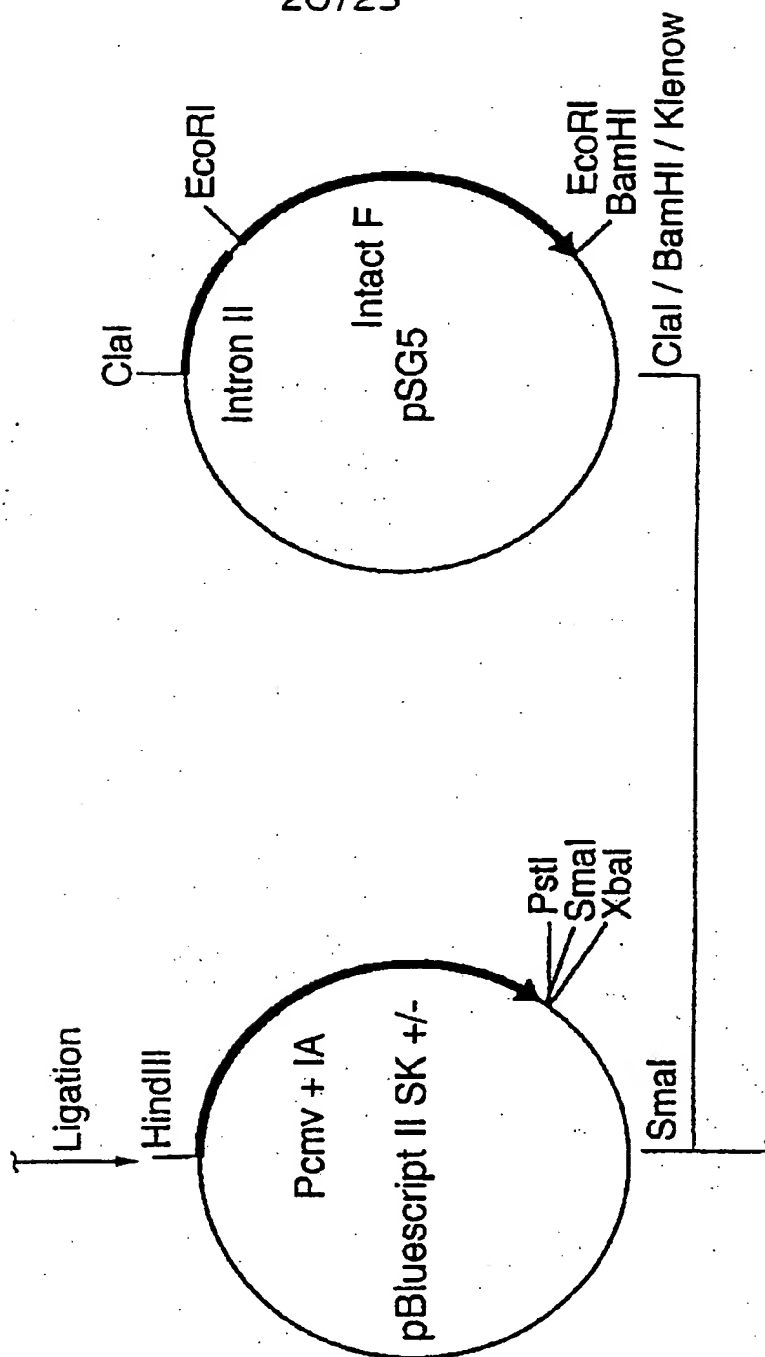


FIG.7B

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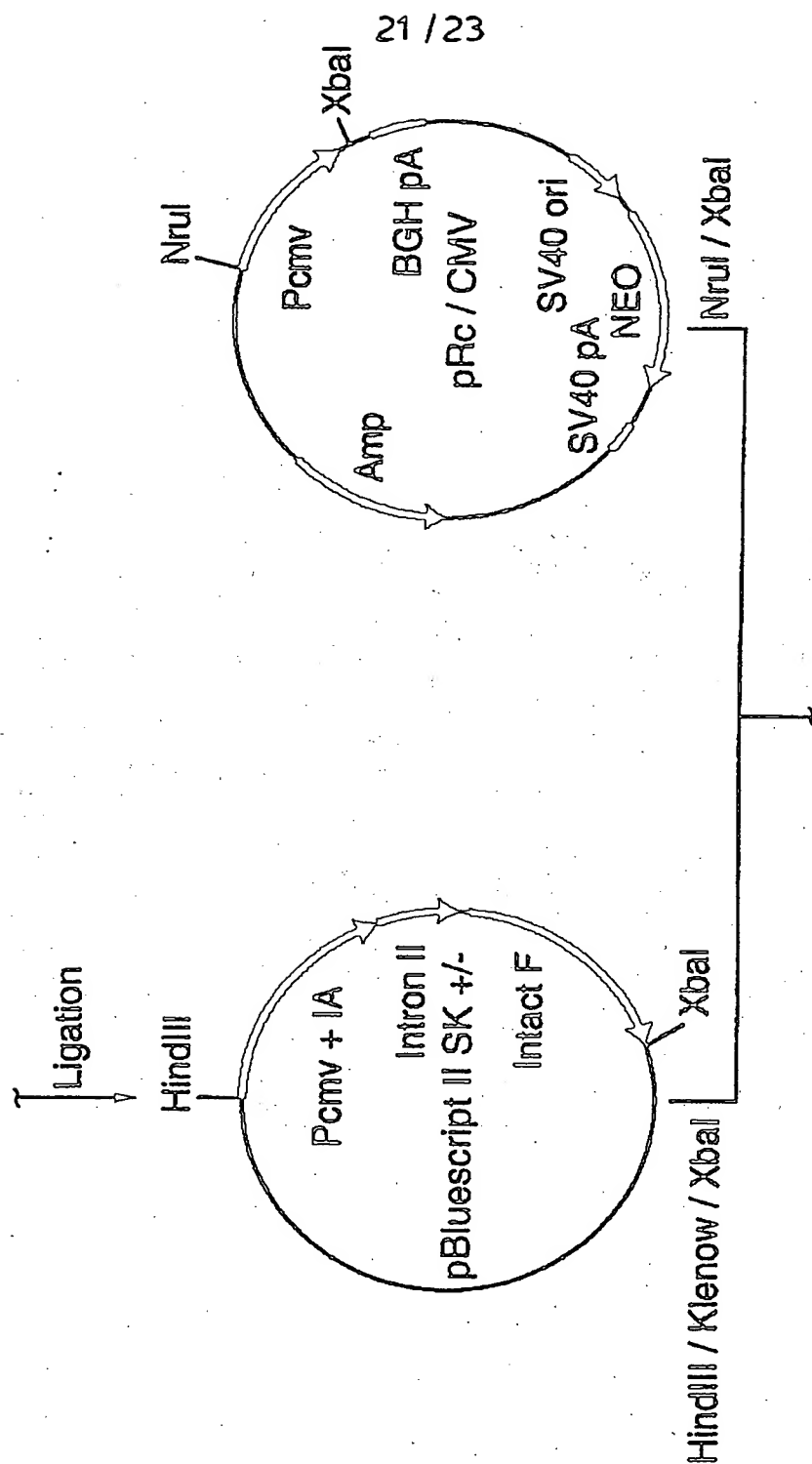


FIG.7C

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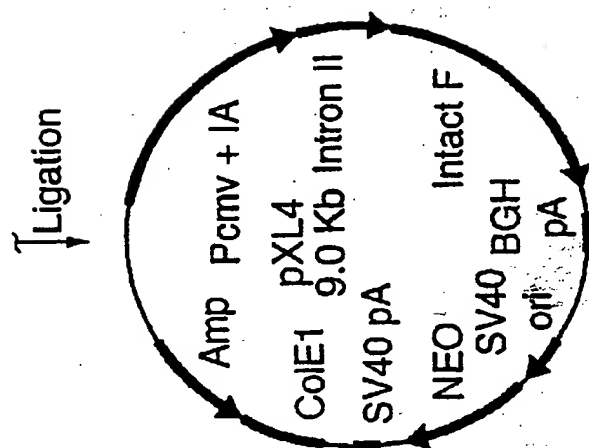


FIG.7D

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401	TTGGGACCC	TTGATTGTTT	TTTCTTTTTC	GCTATTGTAA	AATTCATGTT	GTGAGT
451	ATATGGAGGG	GGCAAAAGTTT	TCAGGGTGTT	GTTTAGAATG	GGAAAGATGTC	
501	CCTTGTATCA	CCATGGACCC	TCATGATAAT	TTTGTTTCTT	TCACCTTTCTA	
551	CTCTGTTGAC	AACCATTTGTC	TCCCTCTTAT	TTCTTTTCAT	TTTCTGTAAC	
601	TTTTTTCGTTA	AACCTTTAGCT	TGCATTGTGA	ACGAATTTT	AAATTCACCT	
651	TTGTTTAATTT	GTCAGATTGT	AAGTACTTTC	TCTAATCACT	TTTTTTTCAA	
701	GGCAATCAGG	GTATATTATA	TTGTACTTCA	GCACAGTTT	AGAGAACAAT	
751	TGTTATAATT	AAATGATAAG	GTAGAAATAT	TCTGCATATA	AATTCCTGGCT	
801	GGCGTGGAAA	TATTTCTTATT	GGTAGAAACA	ACTACATCCT	GGTCATCATC	
851	CTGCCCTTTCT	CTTTATGGTT	ACAATGATAT	ACACTGTTTG	AGATGAGGAT	
901	AAAATACTCT	GAGTCCAAAC	CGGGCCCCCTC	TGCTAACCAT	GTTCATGCGCT	
951	TCCTCTTTT	CCTACAG				

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